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Evolution of Developmental Control Mechanisms

β-catenin plays a central role in setting up the head organizer in hydra

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ABSTRACT

In an adult hydra the head organizer, located in the hypostome, is constantly active in maintaining the structure of the animal in the context of its steady state tissue dynamics. Several *Wnt* genes, *TCF*, and elevated levels of *β-catenin* are expressed in the hypostome as well as during the formation of a new organizer region in developing buds suggesting they play a role in the organizer. Transgenic hydra were generated in which a modified hydra *β-catenin* gene driven by an *actin* promoter is continuously expressed at a high level throughout the animal. These animals formed heads and secondary axes in multiple locations along the body column. Transplantation experiments indicate they have a high and stable level of head organizer activity throughout the body columns. However, none of the *Wnt* genes are expressed in the body columns of these transgenic animals. Further, in alsterpaullone-treated animals, which results in a transient rise in head organizer activity throughout the body column, the time of expression of the *Wnt* genes is much shorter than the time of the elevated level of head inducing activity. These results for the first time provide direct functional evidence that *β-catenin* plays a crucial role in the maintenance and activity of the head organizer and suggest that Wnt ligands may be required only for the initiation but not in maintenance of the organizer in *Hydra*.

Introduction

Organizers, or organizing centers, play an important role in the early stages of embryonic development in a number of metazoans. A similar situation exists in adult hydra, where an organizer is involved in axial patterning to maintain the structure of the adult in the context of its tissue dynamics. The epithelial cells of the ectoderm and endoderm of the body column are constantly in the mitotic cycle (Campbell, 1967a; David and Campbell, 1972) resulting in the displacement of the epithelial tissue apically along the upper part of the body axis into the head, and basally in the lower part into the foot (Campbell, 1967b). At both ends the epithelial cells differentiate and subsequently move towards and are sloughed at the extremities. To maintain the morphology and axial distribution of differentiated cells in this steady state of production and loss of tissue, axial patterning processes are constantly active. These processes are controlled by an organizer region, termed the head organizer, which is located in the hypostome, the apical half of the head of a hydra (Broun and Bode, 2002).

A defining characteristic of an organizer region is its ability to induce a second axis when transplanted to another embryo. Similarly, transplantation of the hypostome of an adult hydra to the body column of a host hydra results in the induction of a second axis consisting of tissue of the host (Browne, 1909; Yao, 1945; Technau et al., 2000, Broun and Bode, 2002). No other part of the animal has this capacity.

In the embryos of several vertebrates [e.g., frog (Tao et al., 2005), zebrafish (Kelly et al., 2000), chick (Boettger et al., 2001), mouse (Mohamed et al., 2004)], β-catenin plays a critical role in setting up the organizer. It is also involved in axial patterning in the sea urchin embryo (Croce and McClay, 2006) and in adult planarians (Gurley et al., 2008). In embryos of *Xenopus laevis*, this activity appears to be associated with the canonical Wnt pathway (Tao et al., 2005). Similarly, two lines of evidence indicate that this pathway is involved in the head organizer in hydra. (1) *HyWnt-3*, the *Hydra* orthologue of *Wnt-3*, which is known to activate the canonical Wnt pathway in vertebrates (Croce and McClay, 2006), is expressed exclusively in the apical portion of the hypostome of an adult hydra (Hobmayer et al., 2000). During bud formation, hydra's mode of asexual reproduction, expression of this gene starts in the apical tip of the developing bud at a very early stage when head organizer synthesis begins (Li and Yao, 1945), and remains there throughout the development of the bud. Two other genes of this pathway, *Tcf* and *β-catenin*, are also expressed in adult hydra. *HyTcf*, the hydra orthologue of *Tcf*, is restricted to the head of the adult as well as in developing buds, but in a broader pattern than *HyWnt-3* (Hobmayer et al., 2000). *Hyβ-cat* is expressed fairly uniformly throughout the adult (Hobmayer et al., 2000). However, only in the hypostome of an adult hydra, is the β-catenin

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protein located in the nucleus (Broun et al., 2005). (2) Treatment of hydra with alsterpaullone, a specific inhibitor of GSK-3β (Leost et al., 2000), resulted in an elevated level of β-catenin protein in the nuclei of cells throughout the body column, and conferred head organizer capacity on the tissue of the body column (Broun et al., 2005). This suggests that β-catenin plays a role in the head organizer.

The recent development of transgenic hydra (Wittlieb et al., 2006) provides a means for directly addressing the role of β-catenin in head organizer formation and activity. Here we describe the use of transgenic hydra overexpressing a β-catenin-eGFP transgene driven by a hydra actin promoter in the epithelial cells of adult hydra, and its relationship to the head organizer. In addition, we provide evidence that Wnt ligands may be required for the initiation, but not the maintenance of the organizer.

Materials and methods

Hydra and culture conditions

Experiments were carried out with 1-day starved animals of three different strains: the AEP and L2 strains of *Hydra vulgaris*, and the 105 strain of *Hydra magnipapillata*. Animals were fed three times per week and maintained in hydra medium as described previously (Smith et al., 1999).

Generation of transgenic hydra

Generation of the β-catenin/GFP fusion construct

A cDNA encoding a version of the *Hydra magnipapillata* β-*catenin* gene lacking amino acids 1-138 was cloned into the pHyvec4 vector (Steele; GenBank accession number DQ385853), which is a modified version of the Hot-G Bluescript II SK+ vector (Wittlieb et al., 2006). *Nhe*I and *Sma*I sites were added to the truncated β-*catenin* cDNA by PCR. The resulting product was inserted into a *Nhe*I/*Sma*I-cleaved pHyVec4 plasmid downstream of the hydra actin promoter and upstream of the *GFP* gene as a carboxy-terminal fusion. The construct was sequenced to confirm its structure. To demonstrate that the construct produced a GFP fusion protein, it was introduced into cells of adult hydra by particle bombardment with the Bio-Rad Biolistic PSD-1000/He Particle Delivery System.

Generation of transgenic hydra

Male and female animals of the AEP strain were cultured together at a high density, and fed 4×/wk for 4 weeks. Thereafter, they were fed normally 2–3 times per week for 3–4 weeks, and they began producing sperm and eggs resulting in fertilized embryos. Embryos at the 2–4 cell stage were injected with 1 nl of 1.3 μg/μl plasmid DNA in 0.2 mM Tris buffer, pH 8.0. Within 3–4 weeks, the embryos that survived injection hatched. Subsequently animals with ectopic heads along their body columns were cultured as described above for normal animals.

Tissue manipulations

Transplantation experiment

The inductive capacity of body column tissue of a transgenic animal was assayed as described previously (Broun and Bode, 2002). This involved isolating a piece of the body column tissue of a transgenic animal similar in size to an eighth of the body column of a normal hydra, cutting it into four equal sized pieces, and transplanting one piece into the body column of host animal labeled with India Ink (Campbell, 1973).

Treatment with alsterpaullone

Animals of the L2 strain of *Hydra vulgaris* were exposed to 5 μmol alsterpaullone (A.G. Scientific, Inc.) for 2 days, and then returned to hydra medium as described previously (Broun et al., 2005). Periodically after begin of treatment, samples were examined for expression of a specific gene using in situ hybridization on whole mounts.

In situ hybridization

In situ hybridization analysis was carried out on whole mounts of hydra as described previously (Grens et al., 1996; Martinez et al., 1997). The antisense RNA probes for *HyWnt-3* [0.1 ng/μl], *HyBra* [0.05 ng/μl], *HyTcf* [0.03 ng/μl] are those described previously (Broun et al., 2005). The probe for *Hyβ-Cat* [0.3 ng/μl] was generated from a β-catenin gene isolated from the L2 strain of *Hydra vulgaris*, while EST or genomic sequences of *Hydra magnipapillata* were used to generate the other probes for the other *HyWnt* genes. The procedure for generating the probes was described previously (Broun et al., 2005). These *Wnt* probes were used at concentrations of 0.03 ng/μl for *HyWnt-1*, *-7*, *-9/10a*, *-11* and 0.1 ng/μl for *HyWnt-3*, *-16*. For all probes, the staining step was carried out for 60 min.

Results

β-catenin transgenic hydra produce multiple heads and axes

To generate a β-catenin transgenic hydra, a pHyvec4 plasmid containing a hydra *actin* promoter/*GFP* construct was modified to contain the construct *actin* promoter/β-*catenin*/*GFP*. The N-terminal part of β-catenin contains the GSK-3β phosphorylation site which regulates the stability of β-catenin protein (Hagen and Vidal-Puig, 2008). To minimize the degradation of β-catenin, the 5`-terminal end of the β-*catenin* gene (encoding amino acids 1–138) was removed prior to insertion into the construct.

Previously it had been shown that injection of embryos (n>2000) of the AEP strain of *Hydra vulgaris* with a plasmid containing a hydra *actin* promoter/*GFP* construct (Wittlieb et al., 2006, Khalturin et al., 2007) invariably resulted in hatchlings that developed into normal hydra (Fig. 1A). In contrast, development of some of the embryos injected with the plasmid containing the *actin* promoter/β-*catenin*/*GFP* construct differed markedly from those receiving the control plasmid. Of the 115 embryos injected, 82 hatched, and of these eight had or developed an abnormal morphology. These eight hatchlings were mosaics with patches of epithelial cells expressing β-catenin/GFP fusion protein. The remaining 74 hatchlings did not contain any GFP-positive cells and developed normally.

Usually when a hydra embryo hatches it has the structure of an adult hydra consisting of a single axis with a head, body column and foot (Fig. 1A). Upon feeding, these animals will reproduce asexually by bud formation. This process starts with the appearance of an evagination of the body column wall located about 2/3-rds down the length of the body column. The evagination elongates into a protrusion. Subsequently a head and foot form at the apical and basal ends of the protrusion, and thereafter, the bud detaches. When fed the bud grows into an adult within a week.

The process was quite different in the eight animals, which showed expression of β-catenin/GFP in patches of epithelial cells and which will be referred to as β-catenin transgenic, or β-cat-Tg, animals. Two of the β-cat-Tg animals had already formed a second axis protruding from the main axis by the time of hatching (Fig. 1B). The others appeared normal upon hatching, but instead of undergoing bud formation, began forming heads along the body column (Figs. 1C–E). This occurred not only in the budding zone, but also in other locations along the body column (Fig. 1F). When fed, a body column would form beneath each of these heads resulting in a secondary axis. This behavior continued with an increase in the number of axes, each with a head at the apical end. Subsequently

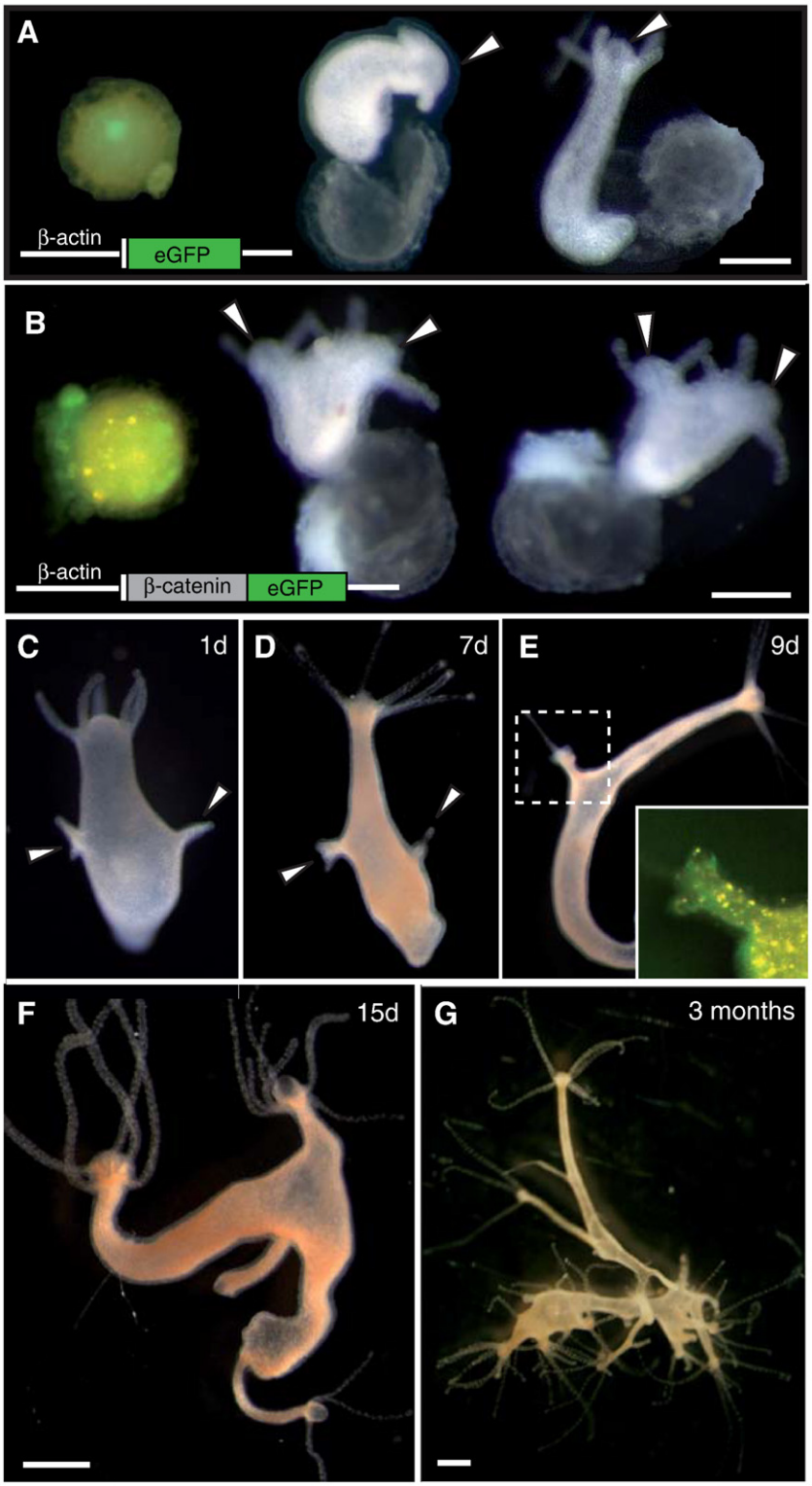


Fig. 1. Development of a β-cat Tg from an embryo to a 3-month-old adult. (A) Hatching of embryos containing the β-actin-GFP control plasmid, and (B) the β-actin–β-catenin-GFP plasmid. (C–G) Development of multiple ectopic heads and second axes in the β-cat Tg with time. (E) The insert indicates the expression of GFP, and hence, β-catenin-GFP in an ectopic head. Bar = 3 mm. Arrowheads in (A–D) indicate position of a hypostome. Yellowish spots in (B) and (E) indicate RITC-dextran which was used as tracer dye during embryo injection. Only bright green fluorescence corresponds to β-catenin-GFP.

new heads would emerge from these secondary axes so that after several weeks each of these animals had generated multiple axes (Fig. 1G).

As a hydra has no defined lifetime, and usually reproduces by bud formation indefinitely, these β-cat-Tg animals have continued to grow over 18 months. They also formed feet as they were firmly attached to the dish in which they were cultured. Occasionally, due to the extraordinary large size of the β-cat-Tg animals two feet formed facing one another in the middle of an unusually long body column resulting in the separation of the animal into two. With hydra's capacity for head regeneration, cutting a β-cat-Tg animal into several pieces resulted in the formation of several of these animals, each of

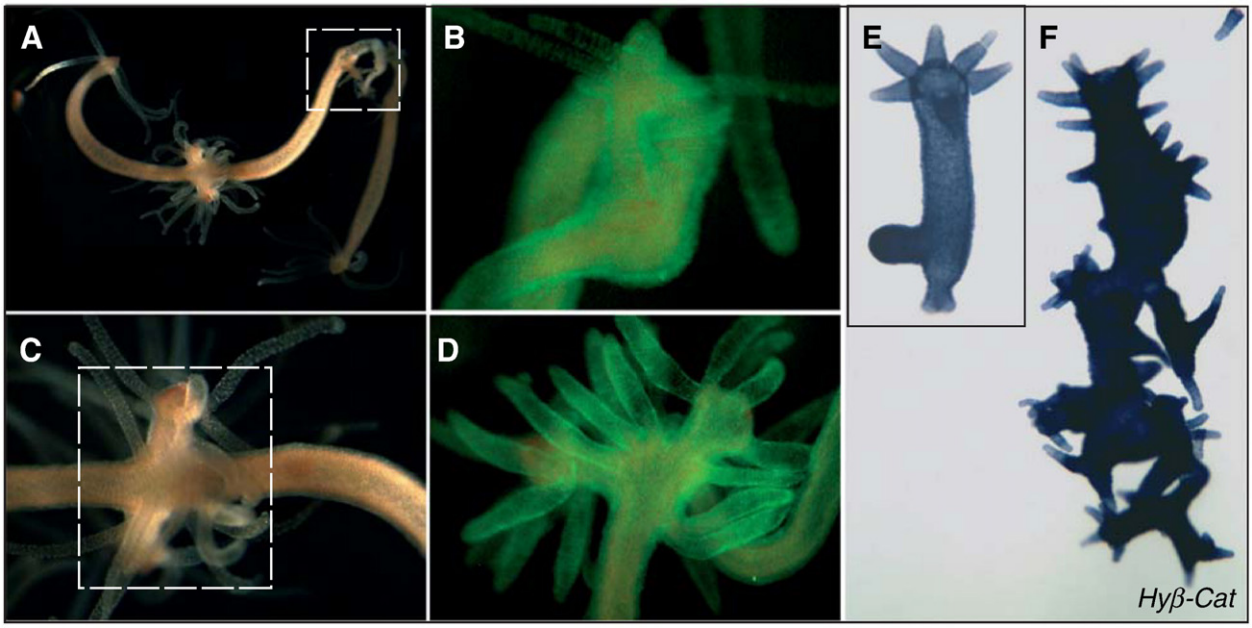
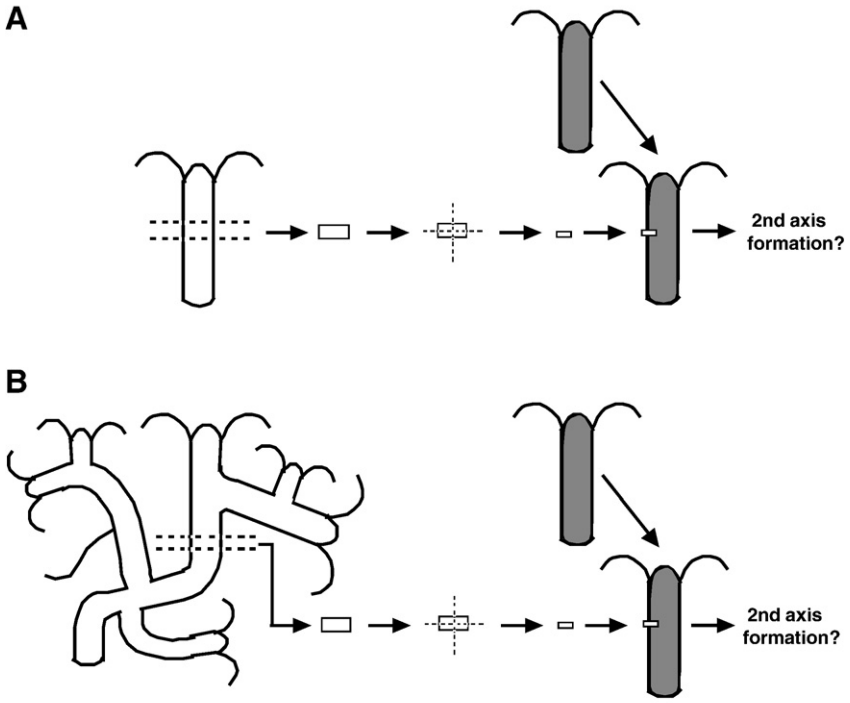


Fig. 2. β-Catenin is strongly overexpressed all over the body in β-cat Tg *Hydra*. (A) A β-cat Tg *Hydra* with several axes. (B-D) Localization of the β-catenin-GFP fusion protein visualized by GFP fluorescence. Regions of the animal shown are indicated by the inserts in (A and C). These photos were taken on a live animal. (E) Expression of *Hyβ-cat* in a normal animal. (F) Expression of *Hyβ-cat* in β-cat Tg *Hydra*.



C

Donor Tissue	Number of Grafts	Type of Axis formed		
		2nd Axis	1-2 tentacles	No dev.
Wild type	45	2	2	41
Beta-cat Tg	45	39	2	4

Fig. 3. Transplantation experiment demonstrating that the body column of β-cat Tg *Hydra* has head organizer capacity. (A) Isolation and transplantation of a piece (1/32nd) of the body column of a normal AEP adult, or (B) a β-cat Tg transgenic AEP animal into the body column of a normal AEP adult. (C) Results of the transplants showing that the body column of the transgenic, but not the normal adult has acquired head organizer capacity.

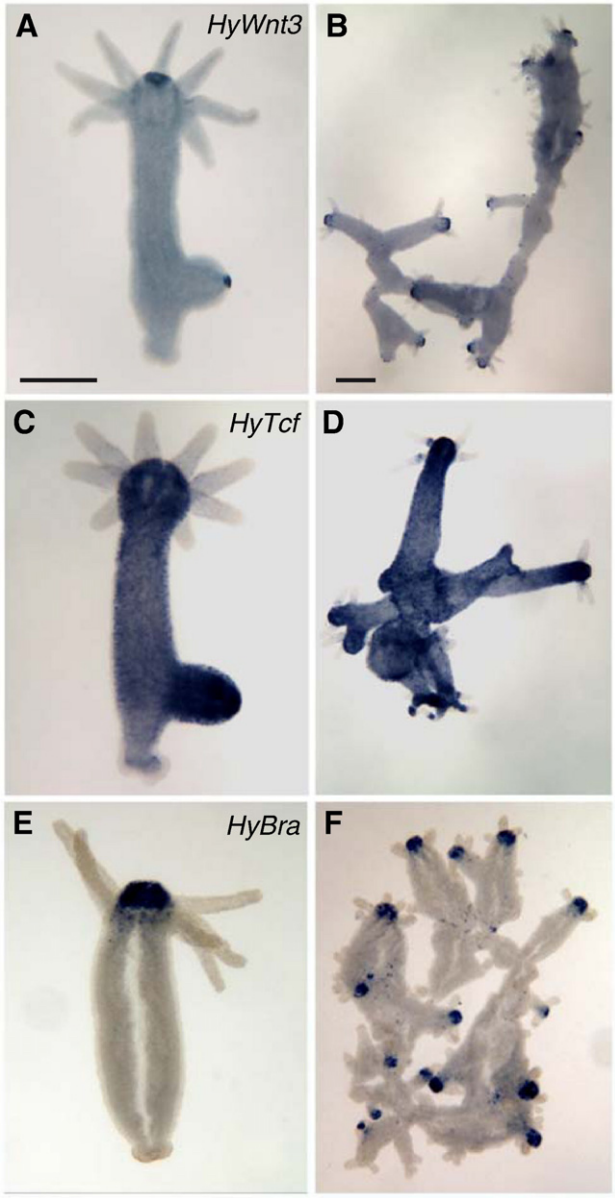


Fig. 4. Expression patterns of *HyWnt-3* (A, B), *HyTCF* (C and D) and *HyBra* (E and F) in control (A, C, and E) and β-cat Tg (B, D, and F) *Hydra*.

which regenerated heads at the cut surfaces continued to grow and expand the number of its axes.

The continuous expression of β-catenin in these animals (Fig. 1E) and (Fig. 2) indicates that the gene plays an important role in the conversion of the morphology from wild-type to this multiple axis phenotype. The expression of the gene started during early stages in embryogenesis as indicated by the presence of GFP in early embryos (Fig. 1B). This expression continued during the formation of the initial secondary axis in a young β-cat-Tg (Fig. 1E), and later in an adult that has begun to form multiple secondary axes (Figs. 2A–D). Several months after the initial generation of these animals, expression of the gene was examined using whole mount in situ. Normally *Hyβ-cat* is expressed at a fairly moderate level uniformly throughout the normal animal with a more elevated level in developing buds (Hobmayer et al., 2000) as shown in Fig. 2E. In contrast, in the transgenic animals, this gene was expressed at a high level throughout the multiple body columns and heads of the animal (Fig. 2F). Hence, the continuous formation of new heads and axes is correlated with a continuously high level of expression of *Hyβ-cat*.

Since the construct was expressed exclusively in epithelial cells and not in the interstitial cell lineage, our results show that overexpression of *β-catenin* in epithelial cells is sufficient to confer a high level of head activation potential on the tissue. This is consistent with earlier observations (Marcum and Campbell, 1978; Sugiyama and Fujisawa, 1978) that patterning processes in hydra are largely driven by epithelial cells. Interstitial cells appear not to be essential for axis formation.

High level of head organizer activity in the body column of transgenic animals

As mentioned above, when the hypostome of a *Hydra* is transplanted to the body column of another hydra, ~90% of the transplants form a secondary axis consisting of a head and body column (Broun and Bode, 2002; Broun et al., 2005). A similar sized piece of body column rarely induces a second axis. The continuous appearance of heads and secondary axes along the body column implies that the tissue of a β-cat Tg body column has the organizer characteristics associated with the hypostome. To examine this possibility, a piece of body column tissue, similar in size to the hypostome, was isolated and transplanted to the middle of the body column of a normal animal. Donor tissue was isolated from either a normal hydra (Fig. 3A) or a β-cat Tg animal (Fig. 3B). As shown in Fig. 3C, <5% of the transplants from normal body columns induced a second axis, while >85% of the transplants from the body columns of β-cat Tg hydra formed a second axis. Thus, the body column of the transgenic animals has an elevated head organizer potency, which most likely accounts for the formation of extra heads and associated axes along the body column of a given axis. As this experiment was carried out with β-cat Tg animals with multiple axes, which were derived from the original transgenic animals, the head organizer characteristics of the body column of these animals appear to be quite stable. The data for the first time provide direct evidence that β-catenin plays a central role in the formation of the head organizer.

The canonical Wnt pathway is involved in the initiation of the formation of the head organizer

In wild-type polyps the continuous expression of *HyWnt-3* and *HyTCF* as well as an elevated level of *Hyβ*-cat in the hypostome where the head organizer is located suggests that the canonical Wnt pathway is continuously involved in the maintenance of the head

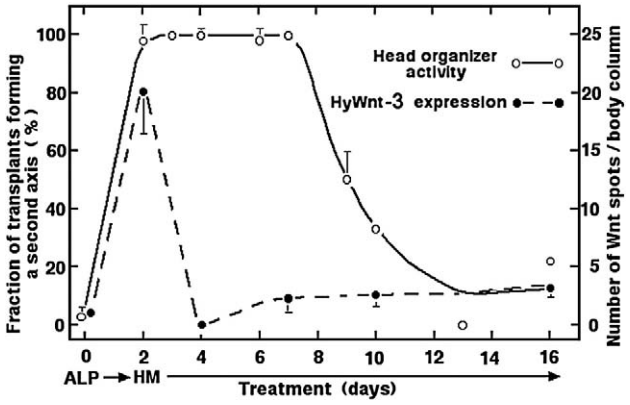


Fig. 5. Relative stability of the head organizer activity and of *Wnt-3a* expression in the body column following treatment with alsterpaullone. For head organizer activity, each data point is the average value ± s.e.m. for 1–3 experiments, and the number of grafts per time point per experiment was 17–28. For Wnt-3a expression, each time point is the average value ± s.e.m. for a sample size of 10. Data points without an error bar are the values for a single experiment.

organizer (Hobmayer et al., 2000). In addition, the expression of *HyBra*, the hydra orthologue of *Brachyury*, is also restricted to the hypostome (Technau and Bode, 1999), which is consistent with the dependence of its expression on the presence of Wnt in *Xenopus* (Vonica and Gumbiner, 2002) and hydra (Bielen et al., 2007). In animals treated with alsterpaullone, which acquire head organizer activity in the body column, *HyWnt-3*, *HyTCF* and *HyBra* are ectopically up-regulated in the body column (Broun et al., 2005). Thus, one might expect these three genes to be highly expressed in the body columns of the β-cat Tg animals. However, this is not the case. As shown in Fig. 4, the expression patterns of these three genes, *HyWnt-3* (Figs. 4A and B), *HyTCF* (Figs. 4C and D) and *HyBra* (Figs. 4E and F) are similar to that in control animals. They are strongly expressed in the hypostomes of β-cat Tg animal, but not at all up-regulated in the body column of transgenic polyps when compared to control polyps. This observation was unexpected and raised the question: are Wnt ligands crucial only for the initiation of the head organizer and not required for the subsequent organizer maintenance?

To address this question, animals were treated with alsterpaullone and analyzed for both head organizer activity and expression of Wnt genes over the period of 16 days (Figs. 5 and 6). Treatment with alsterpaullone conferred head organizer activity on the body column, which remained stable for 6 days, and then gradually declined thereafter (Fig. 5). In contrast, the expression patterns of *HyWnt-3* and *HyBra* were much less stable in these animals. These two genes are strongly expressed in the body column after two days of alsterpaullone treatment (Fig. 5; Figs. 6B and E), but they are no longer expressed two days later (Fig. 5; Figs. 6C and F).

Since it is plausible that one or more of the several other Wnt genes, such as *HyWnt-11* (Lengfeld et al., 2009) might keep the canonical Wnt pathway active, we examined the expression of all other members of the *HyWnt* family that are expressed in the hypostome in alterpaullone treated animals. As shown in Fig. 7, each of these genes is also only transiently expressed for 2–3 days in the body column of an alsterpaullone-treated animal. In contrast, the head organizer activity remains constant for several days after the end of expression of all Wnt genes. These observations indicate that the Wnt ligands are involved in the initial steps of head organizer formation.

Discussion

In the context of the tissue dynamics of an adult hydra (Campbell, 1967b), a mechanism for constantly renewing the head organizer in the hypostome is clearly necessary for its maintenance. One mechanism would involve the canonical Wnt pathway operating in a positive feedback loop as it has been shown to do, for example, in *Drosophila* (Heslip et al., 1997) and mice (Deb et al., 2008). Expression in the apex of the hypostome of one or more *HyWnt* genes (Lengfeld et al., 2009) and *HyTCF* (Hobmayer et al., 2000) is associated with the presence of β-catenin protein in the nuclei of the epithelial cells of this region (Broun et al., 2005). This pattern is consistent with the pathway operating in this manner. Treatment with alsterpaullone provides more direct evidence. A two day treatment with this inhibitor of GSK-3β led to a markedly increased level of β-catenin protein in cell nuclei throughout the body column

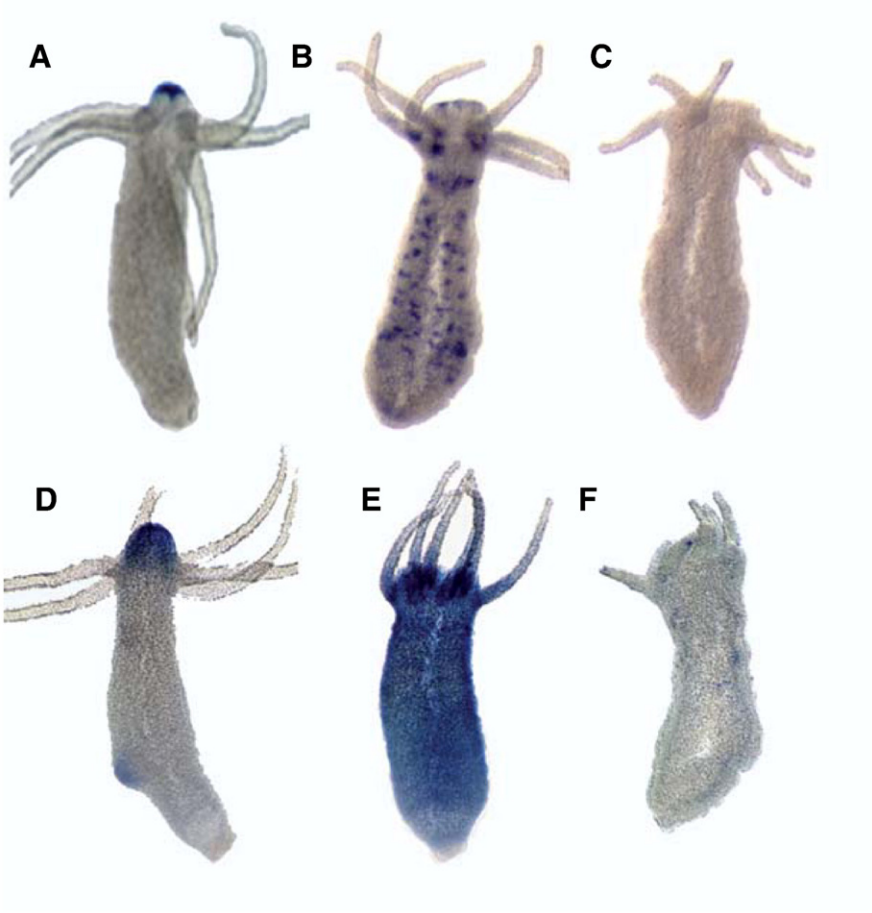


Fig. 6. Rise and decline in the expression of *Wnt-3a* (A, B, and C) and *HyBra* (D, E, and F) in the body column following treatment with alsterpaullone. The time points correspond to (A and D) control, (B and E) 2 days alsterpaullone treatment, and (C and F) 2 days after end of alsterpaullone treatment.

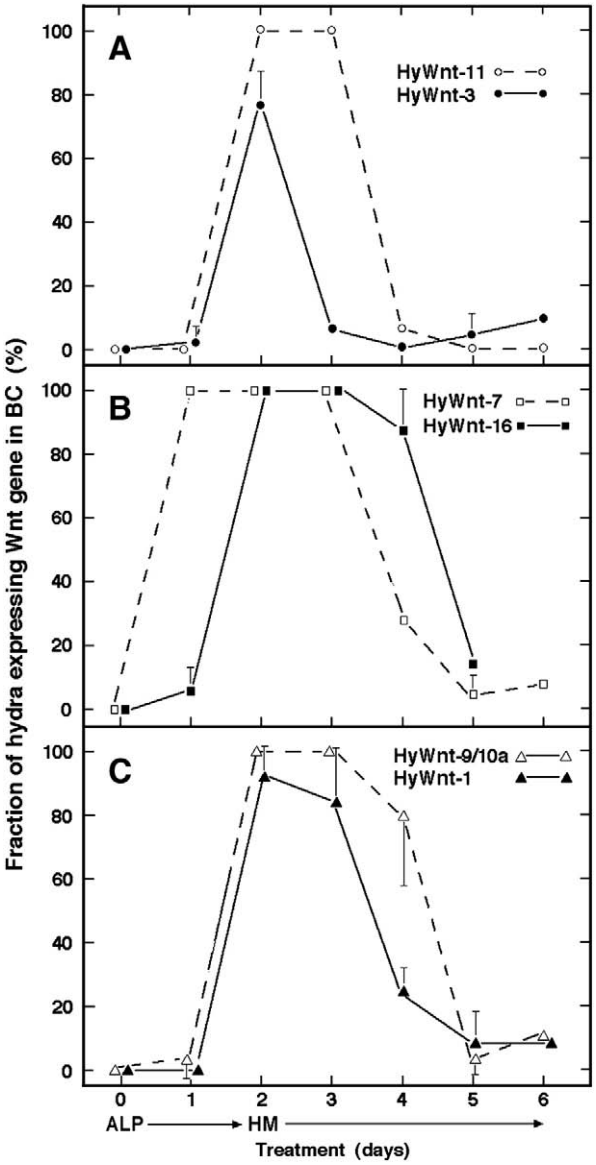


Fig. 7. Effect of treatment with alsterpaullone on the expression of the *HyWnt* genes as spots in the body column. (A–C) each plot represents two of the *HyWnt* genes. Each data point is the average value ± s.e.m. for 2–5 experiments, and the number of grafts per time point per experiment was 9–20. Data points without error bars either had values of 0 or 100, or only 1 experiment was carried out.

(Broun et al., 2005), and also resulted in the expression of both *HyWnt-3* as well as other *HyWnt* genes (see Fig. 7) and *HyTcf* throughout the body column. Thus, the expression of these genes is tightly coupled with the presence of β-catenin in the nucleus, as would be expected if this pathway were acting in a positive feedback loop in the hypostome.

In this context of continuously apically displaced tissue, some of the Wnt protein produced in this feedback loop in the apex of the hypostome would diffuse basally and initiate the activity of the canonical Wnt pathway in these neighboring hypostome cells. This behavior would lead to translocation of activated β-catenin into nuclei, which then would initiate head organizer activity in these slightly more basal cells (Fig. 8). In this manner, the continuous apical displacement and sloughing of head organizer activity at the apex would be balanced by the continuous production of new head organizer activity slightly below the apex. This activity would maintain the head organizer in the context of the tissue dynamics of a hydra.

β-catenin is required for setting up head organizer activity

Tissue of any region of the body column of a normal adult hydra has the potential to form a head organizer. Bisect the animal anywhere along the body column, and a head organizer will form at the apical end of the lower half resulting in the regeneration of a head. Though this tissue has the potential to form a head organizer, it is not determined to do so, as transplantation of a piece of body column tissue similar in size to a hypostome rarely results in the formation of a head and a secondary axis (Yao, 1945; Broun and Bode, 2002). In contrast, a similar size piece of body column tissue of a β-cat Tg animal induces the formation of a secondary axis with a high frequency (Fig. 3). This indicates that tissue expressing a high level of stable β-catenin has a much higher potential for the formation of a head organizer.

However, there are two unexpected features of the β-cat Tg animals, which are not completely understood. [1] Heads, and thus, head organizers, do not form everywhere along the body column of a β-cat Tg animal (Figs. 1F, G). [2] While treatment with alsterpaullone leads to the ectopic expression of *HyWnt*, *HyBra1* and *HyBra2* (Broun et al., 2005; Bielen et al., 2007), the ectopic expression of stabilized β-catenin does not.

One explanation for the first puzzle may be that head organizers, and thus, secondary axes are prevented from forming all along the body column in a β-cat Tg animal by the head inhibitor. In a wild-type animal, the head organizer in a normal animal produces and secretes a head inhibitor, which diffuses down the body column and prevents tissue from undergoing formation of a head organizer, and subsequently head formation (MacWilliams, 1983). As the concentration of head inhibitor is graded down the body column, new axis formation in the budding zone indicates that the level of head inhibition has dropped below an effective inhibitory level. Similarly, in β-cat Tg animals, new heads and subsequently secondary axes will form at some distance from one another where the secreted head inhibitor level of a recently formed head drops below the effective level. When transplanted into a wild-type host, a piece of tissue overexpressing β-catenin may block the influence of the head inhibitor, and successfully initiate secondary axis formation. This may not happen everywhere along the body column of a β-cat Tg animal as the multiple secondary axes may be producing enough head inhibition to override the capacity of β-catenin to block the head inhibitor, and thereby block the initiation of head organizer formation.

The second issue concerning the lack of expression of downstream genes in the transgenic animals is most likely related to the level of *HyTCF* in the β-cat Tg animals. For β-catenin to function as a transcriptional activator in the nucleus requires TCF as a co-activator (Cadigan and Nusse, 1997). In a wild-type hydra, β-catenin is present in the nucleus only in cells where *HyTCF* is strongly expressed. This occurs in the hypostome in wild-type hydra and throughout the body column in alsterpaullone-treated animals (Broun et al., 2005). In the transgenic animals, *HyTCF* is also strongly expressed only in the hypostome. This could explain why the downstream genes such as *HyWnt3* and *HyBra1* are not expressed in the body column of the β-cat Tg animals. Thus, the presence of stabilized β-catenin appears to be a necessary but not sufficient precondition for secondary axis formation.

Taken together, the results indicate that once a high level of β-catenin has been achieved, the Wnt ligand and the canonical Wnt pathway are no longer necessary for the induction of the next steps in the formation of the head organizer.

Head organizer activity is downstream of beta-catenin

In *Xenopus* and zebrafish embryos, β-catenin activity during organizer formation leads to the sequential expression of *goosecoid*, and thereafter, other downstream genes associated with the organizer

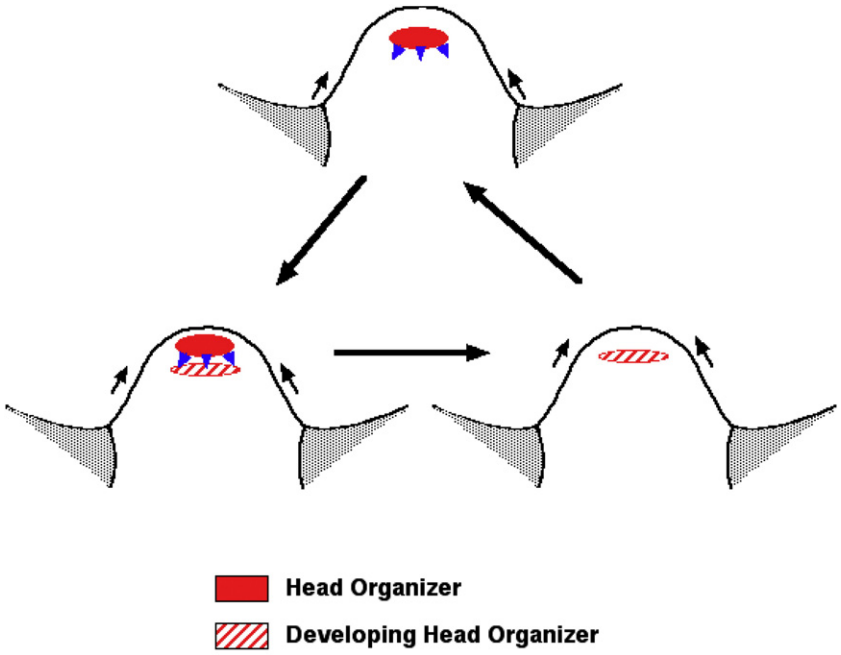


Fig. 8. Production and turnover of the head organizer in the context of the tissue dynamics of the hypostome.

(e.g., Fekeny et al., 1999, Heasman, 2006; Dixon Fox and Bruce, 2009). A related process in terms of the initiation of the expression of genes downstream of β-catenin activity may also occur in hydra. The 2-day alsterpaullone treatment blocking GSK-3β activity led to an elevation of the level of β-catenin protein as well as of *HyWnt-3* expression (Broun et al., 2005). This rise in expression of *HyWnt-3* was transient as it has vanished in the body column 2 days after end of alsterpaullone treatment. One would expect this would lead to a reduction of the level of β-catenin protein in the body column. However, the induced head organizer activity remains at a high level for six days after end of the treatment.

This continuing elevated level of head organizer activity in the alsterpaullone-treated hydra could be due to one of the three effects.

[1] Plausibly one or more of the other six *Wnt* genes, which are also only expressed in the hypostome of normal animals (Lengfeld et al., 2009) is/are involved in the maintenance of head organizer activity in the body column. And, though all of them are also expressed in the body column following alsterpaullone treatment, the expression of each of these genes has vanished or been reduced to very low levels 2–3 days after end of the treatment.

[2] Another possibility is that there is sufficient Wnt protein around after the cessation of the expression of the *Wnt* genes to maintain the activity of the canonical Wnt pathway for the 7 days following the end of alsterpaullone treatment. However, two pieces of evidence suggest this is unlikely: (*a*) experience with Wnt proteins in cell culture indicates they have a half-life of a few hours (Cadigan et al., 1998; Strigini and Cohen, 2000); (*b*) as mentioned above expression of *Brachyury* is dependent on the presence of Wnt proteins associated with the canonical Wnt pathway. *HyBra* is normally expressed in the hypostome (Technau and Bode, 1999) as well as in the body columns of alsterpaullone-treated animals (Broun et al., 2005). And, as with the *HyWnt* genes, the expression of *HyBra* in the body column has ended 2 days after the end of alsterpaullone treatment. Thus, it is unlikely that Wnt protein is present in the body column at this time and subsequently.

[3] Since β-catenin is essential for setting up and maintaining the head organizer, it could be that the elevated level of β-catenin induced by alsterpaullone treatment, was maintained over the seven days following the end of alsterpaullone treatment. This is highly unlikely due to the short half-life of the β-catenin protein, which varies from 0.25 to 4h among *Drosophila* (Pai et al., 1997), sea urchins (Weitzel et al., 2004), *Xenopus* (Yost et al., 1996; Guger and Gumbiner, 2000) and a human cell line (Byers et al., 1996). The half-life for the hydra β-catenin protein is not known, but was it 4 h, then the elevated level would drop back to a normal level within 24 h.

Thus, it is unlikely that the canonical Wnt pathway remained active in the body column for more than 2–3 days after end of alsterpaullone treatment. Therefore, the maintenance of the head organizer for at least 6 days is most likely due to the expression of genes downstream of this pathway. Plausibly it involves a pathway similar to that in *Xenopus* and zebrafish leading from β-catenin through *goosecoid* to other genes of the organizer (Moon & Kimmelman, 1998). In hydra a similar pathway could be involved. Interestingly, *CnGsc*, a hydra goosecoid homologue, is expressed in the hypostome, and even more strongly in the apical end of a developing bud, which has organizer capacity (Broun et al., 1999). Further, its expression in the apex of the developing bud begins after the initial expression of each of the *Wnt* genes.

Hence, as in a number of deuterostomes, β-catenin plays a central role in setting up an organizer region in hydra. And, as in *Xenopus* embryos, the canonical Wnt pathway is involved. This suggests that the application of this pathway for the purpose of setting up an organizer region first appeared early in metazoan evolution, and has been conserved among a number of deuterostomes.

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